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Comparison of Bacterial Cellulose Production in a Jar Fermentor Between *Acetobacter xylinum* BPR2001 and its Mutant, Acetan-Nonproducing Strain EP1

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Abstract The bacterial cellulose (BC) production by a wild-strain *Acetobacter xylinum* BPR2001 and that by its acetan-nonproducing mutant, EP1, were compared in a jar fermentor. EP1 produced about 28% less BC than the wild-strain. The apparent difference in the cultivation of the two strains was the viscosity increase in the culture broth that was closely associated with acetan production. Increasing the viscosity of the culture broth of EP1 by adding agar led to the formation of relatively small and uniform BC pellets, and BC production consequently became two-fold higher than that in the absence of agar and was almost equal to that by BPR2001. Therefore, acetan has an important role in BC production by inducing physical changes in the culture broth of the wild-type strain.

Key words: *Acetobacter xylinum*, acetan, bacterial cellulose, viscosity

Using various sugars, *Acetobacter xylinum* can synthesize insoluble bacterial cellulose (BC) that is secreted into the medium, and this cellulose forms a clump of microfibrils [1, 2]. Because BC has unique physical properties, interest in its mass production for industrial applications is growing [3, 4]. *A. xylinum* BPR2001 was isolated as a high BC producer under agitated culture conditions [5], and the BC production by BPR2001 was found to be higher in stirred-tank and airlift reactors than in conventional static reactors [6, 7]. This strain also produces an anionic water-soluble polysaccharide called acetan, whose chemical structure is similar to xanthan [8, 9]. Studies on the genetic analysis of the biosynthetic pathway and on the chemical structure of acetan by NMR have been reported [10], and studies on the genetic and metabolic bases of BC synthesis have also been carried out [11, 12]. However, no clear relationship between BC and acetan production has been established.

We have been interested in the genetic regulation of the pathways involved in the synthesis and excretion of BC and acetan, and recently generated acetan-nonproducing mutant EP1 from *A. xylinum* BPR2001 [13, 14]. We speculated that the BC production rate of EP1 might increase, because this mutant EP1 does not synthesize the byproduct acetan, therefore, carbon source could be used in BC synthesis, or the supply of UDP-glucose, a direct precursor of BC, could increase the synthesis.

In this study, we investigated the role of acetan by comparing BC production between the wild strain and the acetan-nonproducing mutant in a jar fermentor without oxygen limitation.

Materials and Methods

Microorganisms

The acetan-nonproducing mutant EP1 was generated from the wild-strain, *A. xylinum* BPR2001 [5], by disruption of the *aceA* gene, whose protein product catalyzes the first step of the acetan biosynthetic pathway in the wild-type strain, as previously reported [13, 14].

Culture Conditions

The culture medium used in this study was CSL-Fru medium containing (per liter of deionized water) corn steep liquor (CSL) (Nihon Starch Industry, Japan) 20 ml; fructose 40 g; KH_2PO_4 1 g, $MgSO_4 \cdot 7H_2O$ 0.25 g, $(NH_4)_2SO_4$ 3.3 g, $FeSO_4 \cdot 7H_2O$ 3.6 mg, $CaCl_2 \cdot 2H_2O$ 14.7 mg, $NaMoO_4 \cdot 2H_2O$ 2.42 mg, $ZnSO_4 \cdot 7H_2O$ 1.73 mg, $MnSO_4 \cdot 5H_2O$ 1.39 mg, $CuSO_4 \cdot 5H_2O$ 0.05 mg, and vitamin solution 10 ml. The vitamin solution consisted of inositol 200 mg, nicotinic acid 40 mg, pyridoxine hydrochloride 40 mg, thiamine hydrochloride 40 mg, D-pantothenic acid calcium 20 mg, riboflavin 20 mg, *p*-

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aminobenzoic acid 20 mg, folic acid 0.2 mg, and D-biotin 0.2 mg (per liter of deionized water). The pH of the medium was adjusted to 5.0. An antifoaming agent (Disfoam GD, Nihon Yushi Co., Ltd., Tokyo, Japan) was used to prevent formation of foam on the surface of the medium in a jar fermentor cultivation.

The preculture was performed in a 750-ml Roux flask which contained 100 ml of CSL-Fru medium after addition of the cell suspension which was obtained by filtration of the cellulose pellicle formed on the surface of the CSL-Fru broth medium statically cultivated at 30°C for 3 days. For shaking-flask cultivation, 12.5 ml of the cell suspension containing approximately 1×10^8 cfu/ml were inoculated into 125 ml of the medium in a 500-ml slant baffled flask and cultivated in a rotary shaker (Bioshaker BR-3000L, Taitec Co., Ltd., Japan) at 30°C and 180 rpm. *A. xylinum* BPR2001 and the mutant EP1 were also cultivated in a 10-l jar fermentor (BMS-10PI, Biott Co., Japan) (185 mm diameter and 390 mm height) equipped with two turbines 92.5 mm in diameter. The turbines were constructed with four and six baffle plates, respectively. The 500 ml culture broth obtained from flask cultivation was homogenized (Excel Auto Homogenizer, Nihonseiki Co., Ltd., Tokyo, Japan) at 10,000 rpm for 1 min and inoculated into 4.5 l of sterilized CSL-Fru medium in a 10-l jar fermentor (working volume 5 l). The temperature of the reactor was maintained at 30°C, and the pH was maintained at 5 by the automatic addition of 4 N NaOH or 4 N H_2SO_4. Filtered air was supplied at 2 l/min (0.4 vvm). The dissolved oxygen (DO) concentration of the broth was maintained at about 30% saturation in CSL-Fru medium at 30°C by changing the agitation speed. During cultivation, oxygen and carbon dioxide concentrations in the exhaust gases were continuously online monitored using an oxygen-carbon dioxide meter (EX-1562-1, Able, Japan).

Analytical Methods

Concentrations of BC and Acetan. The culture broth obtained from a 10-l jar fermentor was homogenized at 10,000 rpm for 1 min and centrifuged at 4,000 rpm for 20 min (J6-HC, Beckman, U.S.A.). The precipitated pellets were treated with 0.1 N NaOH solution at 80°C for 20 min to remove bacterial cells and medium components. The cellulose pellets were then rinsed with deionized water. This procedure was repeated three times, and purified cellulose was dried at 80°C in a vacuum oven for 8 h and then weighed. The amount of water-soluble polysaccharide acetan secreted in the medium by BPR2001 was measured by the phenol-sulfate method as previously reported [6]. The acetan, consisting of glucose, mannose, glucuronic acid, and rhamnose in a molar ratio of 4:1:1:1, was converted into the monosaccharide components of acetan, and then acetan concentration was calculated from the optical density at 492 nm using a glucose standard curve as reference.

Concentrations of Fructose and Lactate. The concentration of fructose, the main carbon source in CSL-Fru medium, was determined by high-performance liquid chromatography (HPLC). Samples were centrifuged at 4,000 rpm for 20 min, and the supernatant was then diluted three-fold with ethanol. After filtration through a 0.2 μm filter (ADVANTEC, Japan), fructose concentration was determined with an ODS Shodex NH2P-50 4E column (Showa Denko Co., Ltd., Tokyo) at 40°C and a mixture of acetonitrile and deionized water (=3:1, v/v) as eluent at a flow rate of 1 ml/min. The concentration of lactate in the CSL was calculated from the absorbance at 540 nm with lactate reagent (SIGMA DIAGNOSTICS, Inc.) [15]. The concentrations of fructose and lactate in the final samples were also used in the carbon mass balance calculation.

Number of Viable Cells. The number of cells in the broth was measured from the total cell number which includes cells entrapped in the BC pellicle and suspended in the culture broth and from the number of free cells which contains only cells suspended in the culture broth. The total cell number was determined by the plate dilution method as colony forming units (cfu) after treatment of the culture broth with cellulase (Celluclast, Novo Nordisk A/S, Denmark) at 30°C for 1 h to hydrolyze the BC pellicle. The number of free cells was also determined from the number of cfu of the cells suspended in the culture broth after the culture broth was centrifuged to remove the BC pellicle.

ATP Concentration of Total Cells. The intracellular ATP concentration of the total cells was quantitatively determined using a bioluminescence detection kit with firefly luciferase (Promega, U.S.A.). ATP was extracted from the cells with 2% trichloroacetic acid (TCA) using the manufacturer's protocol, and luminescence produced was measured using a bioluminometer (TD-20/20 luminometer, Turner Designs, U.S.A.). ATP concentration was converted to ATP content of one unit volume of culture broth (mole ATP per ml).

Concentration of Gluconate. The concentration of gluconate was determined using a gluconate detection kit (Roche, Germany).

Measurement of Oxygen Uptake Rate (OUR). OUR is defined as the amount of oxygen consumed per unit volume of culture broth per hour. This was determined from the difference between the inlet oxygen concentrations and outlet oxygen concentrations which were monitored continuously.

Relative Viscosity of Culture Broth. After centrifugation of the culture broth, the cells and BC were removed, and the viscosity of the supernatant was measured with an Ostwald viscometer (Sibata Scientific Technology Ltd., Japan) at 30°C. The viscosity of each culture broth sample was calculated as relative viscosity against distilled water at 30°C.

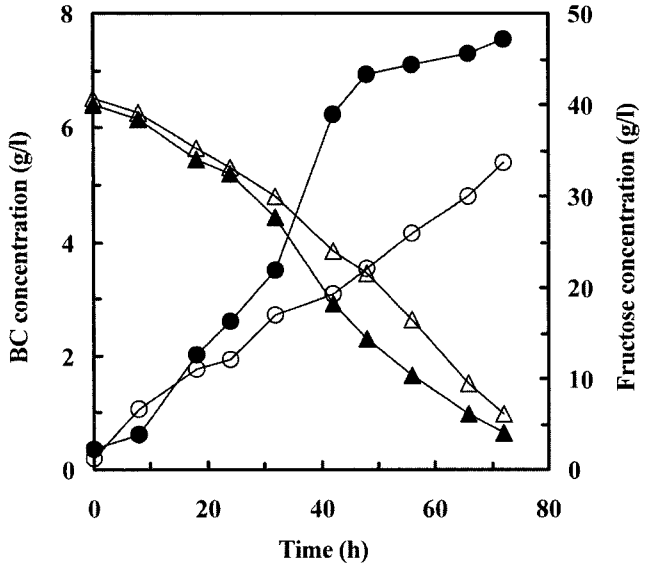

Fig. 1. Bacterial cellulose production and fructose consumption by *A. xylinum* BPR2001 and acetan-nonproducing mutant EP1 for batch cultivation in a 10-l jar fermentor.
The closed circles and triangles indicate bacterial cellulose and fructose concentration of *A. xylinum* BPR2001, respectively. The open circles and triangles indicate bacterial cellulose and fructose concentrations of the acetan-nonproducing mutant EP1, respectively.

RESULTS

BC Production by *A. xylinum* BPR2001 and Acetan-Nonproducing Mutant EP1

The BC production and fructose consumption by *A. xylinum* BPR2001 and the mutant EP1 in a 10-l jar fermentor cultivation are shown in Fig. 1. Up to 20 h, the rates of BC production and fructose consumption of the two strains were similar, however, after 20 h, the BC production rate of the wild-strain increased markedly, compared with that of EP1, followed by a higher fructose consumption rate than that of EP1.

Table 2. Calculated factors from the data in Table 1.

Factors	BPR2001	Mutant EP1
ΔC^a (mol)	5.99	5.65
ΔP^b (mol)	5.68	4.92
$\Delta P/\Delta C$	0.95	0.87
$\Delta BC^c/\Delta C$	0.21	0.16
$\Delta Biomass^d/\Delta C$	0.11	0.10
$\Delta CO_2^e/\Delta C$	0.54	0.60

The values calculated by carbon-mol basis after 72 h cultivation.
[a]Total amount of consumed carbon source of fructose.
[b]Total amount of carbon-containing products.
[c]Total amount of BC produced.
[d]Increased amount of biomass.
[e]Total amount of CO_2 evolved.

As shown in Table 1, the carbon mass balance of the wild-strain and mutant EP1 was constructed on the basis of concentrations of carbon in the products against the carbon of fructose and lactate consumed in CSL-Fru medium. The BC, biomass, and acetan obtained after 72 h of cultivation were dried in vacuum at 80°C for 8 h, and their elemental compositions were analyzed using an elemental analyzer (MT-5, Yanaco, Japan). Only gluconate was quantified as organic acid product. The elemental compositions (per C atom) of the compounds used were as follows: fructose, CH_2O; lactate, CH_2O; BC, CH_2O; biomass, $CH_{1.86}O_{0.4}N_{0.22}$; acetan, $CH_{1.78}O_{0.85}$; and gluconate, $CH_2O_{1.17}$.

The results of the carbon mass balance for the two strains showed that 89.3% and 81.7% of the carbon inputs were recovered as carbon output in the wild-strain and the mutant EP1, respectively. The recovery rate (89.3%) of carbon in BPR2001 was almost the same as that previously reported in airlift reactor cultivation in our laboratory [6]. A lower recovery of carbon (81.7%) in the mutant EP1 might have been caused by the presence of other by products such as organic acids. Table 2 shows the parameters calculated from the data listed in Table 1, when only fructose as a carbon source was taken into consideration.

Table 1. Carbon mass balance for the 10-l jar fermentor cultivation of *A. xylinum* BPR2001 and acetan-nonproducing mutant EP1.

Components	Input		Output[a]			
	Conc. (g/l)	Carbon (mol)	Conc. (g/l)		Carbon (mol)	
			BPR2001	EP1	BPR2001	EP1
Fructose	40	6.67 (94.6%)[b]	4.1	6.1	0.68 (10.7%)[b]	1.02 (17.1%)[b]
Lactate	2.3	0.38 (5.4%)	0.048	0.053	0.008 (0.1%)	0.009 (0.2%)
BC			7.55	5.4	1.26 (19.8%)	0.91 (15.3%)
Biomass			3.1	2.71	0.66 (10.4%)	0.58 (9.7%)
Acetan			2.8	0	0.51 (8.0%)	0
Gluconate			0.1	0.33	0.02 (0.3%)	0.05 (0.8%)
CO_2			28.4	29.7	3.23 (50.7%)	3.38 (56.8%)
Total		7.05 (100.0%)			6.37 (100.0%)	5.95 (100.0%)

[a]The data were obtained after 72 h cultivation.
[b]The values in the parenthesis indicate percentage of each component for the total values.

The ratios of carbon conversion to products (ΔP/ΔC) and BC yield (ΔBC/ΔC) of the wild-strain were larger than those of the mutant EP1, and the yields of biomass (ΔBiomass/ΔC) were similar between the two strains. Although the BC yield of the mutant (0.16) was lower than that of the wild-strain (0.21), the production of gluconate and carbon dioxide by EP1 was larger than that by the wild-strain. Both the wild and the mutant EP1 strains consumed more than half of the carbon to produce carbon dioxide.

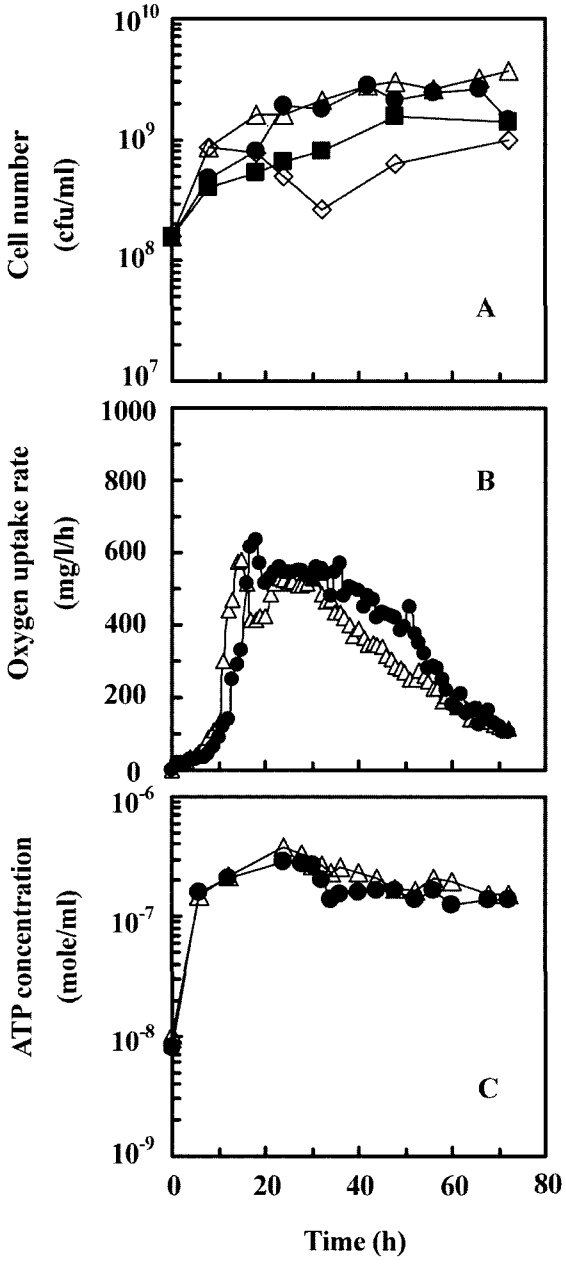

Fig. 2. Changes in cell number (A), oxygen uptake rate (OUR) (B), and intracellular ATP concentration (C) of *A. xylinum* BPR2001 and acetan-nonproducing mutant EP1 for batch cultivation in a 10-l jar fermentor.

A: The closed circles and squares indicate the total cell and free cell numbers of *A. xylinum* BPR2001, respectively. The open triangles and diamonds indicate total cell and free cell numbers of the acetan-nonproducing mutant EP1, respectively. B: The closed circles and open triangles indicate the OURs of *A. xylinum* BPR2001 and acetan-nonproducing mutant EP1, respectively. C: The closed circles and open triangles indicate ATP concentrations of *A. xylinum* BPR2001 and acetan-nonproducing mutant EP1, respectively.

Figure 2A shows the changes in total cell number, which includes suspended cells in the culture broth, BC-entrapped cells, and the number of free cells which includes only the cells suspended in the broth. Although the initial growth of the mutant EP1 seemed to be faster than that of the wild-strain, the total cell numbers of the two strains were almost the same after 20 h. After 24 h, however, the number of free wild-type cells became significantly larger than that of mutant EP1 cells and, as shown in Fig. 1, the BC production by the wild-strain rapidly increased and reached about 7.6 g/l in 72 h. On the other hand, the number of free mutant EP1 cells was about three times lower than that of wild-type strain cells after 32 h, and this difference in the cell number was reflected by the difference in BC production.

Oxygen Uptake Rate (OUR) and ATP Production

ATP content and OUR are considered to be indicators of cell activity [16]. Figure 2B shows the change of OUR by the two strains. OUR rapidly increased during the growth of the two strains, and thereafter gently decreased. This pattern of BC production is typical when lactate is added to the medium [17]. In the present study, although the maximum OURs of the wild-strain and mutant EP1 reached 636 mg O_2/l/h and 580 mg O_2/l/h, respectively, the total amounts of oxygen taken up by the two strains, calculated by the integration of OUR patterns, were not significantly different.

As shown in Fig. 2C, when the intracellular ATP concentrations of the two strains were determined to compare the metabolic activity of the cells [18, 19], there was no considerable difference in ATP concentration between the culture broth of the two strains, reflecting similar trends in the total cell numbers of the two strains. This indicates that the mutant EP1 does not synthesize the by product acetan, therefore, the ATP produced by the EP1 should have been utilized in the production of other metabolites. This was partly confirmed by the fact that the mutant EP1 produced more gluconate and carbon dioxide than the wild-type strain.

Effect of Acetan on BC Production

The significant difference in the cultural characteristics between the wild-type strain BPR2001 and the mutant EP1 was the increase in viscosity of the culture broth, as shown in Fig. 3. Acetan production by the wild-type strain increased markedly after 24 h and reached 2.8 g/l after 72 h, and the viscosity of the culture broth of the wild-strain increased

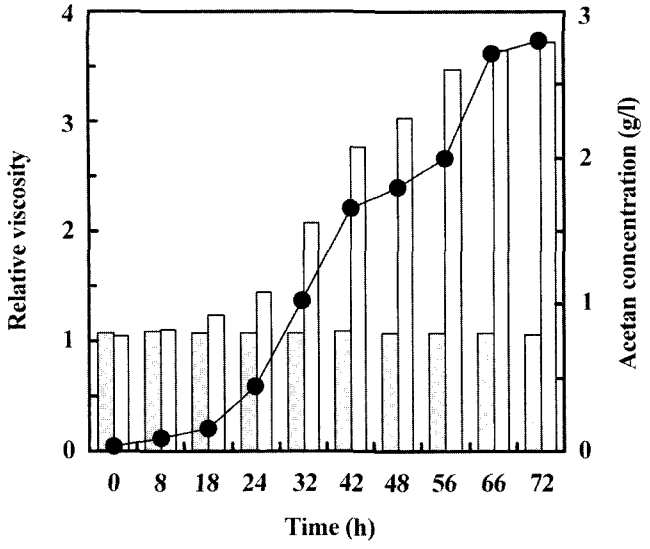

Fig. 3. Concentration of acetan (closed circles) produced by *A. xylinum* BPR2001 and the relative viscosity of culture broth of *A. xylinum* BPR2001 (open bars) and acetan-nonproducing mutant EP1 (closed bars).
Relative viscosity was calculated using the viscosity of distilled water as 1.0.

accordingly and was about 3.7 times higher than that of the mutant EP1 after 72 h of cultivation. The viscosity of the culture broth of the mutant EP1 was almost the same as that of distilled water. Figure 4 shows the change in the morphology of the BC pellets formed during cultivation in a 10-l jar fermentor. The BC pellets from the wild-strain gradually became more uniform in accordance with the increase in acetan production. However, the BC produced by mutant EP1 clumped irregularly. The BC pellets formed by the wild-strain was smaller on average than those formed by the mutant EP1. The results indicate that the viscosity of the culture broth affects the size and uniformity of BC pellets.

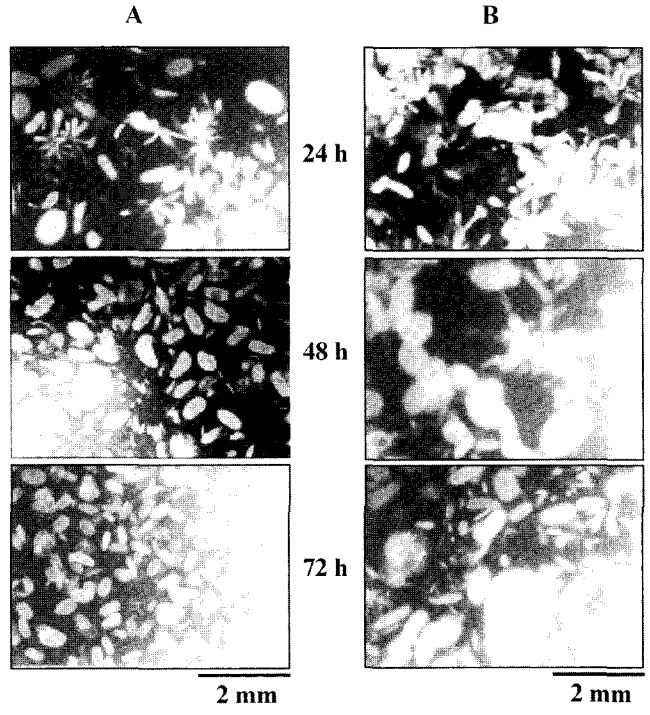

Fig. 4. Morphology of bacterial cellulose produced by *A. xylinum* BPR2001 (A) and acetan-nonproducing mutant EP1 (B) after 24, 48, and 72 h cultivations in a 10-l jar fermentor.

DISCUSSION

BC Production of Acetan-Nonproducing Mutant EP1

Several efforts to improve BC production, such as establishing an economical production process for cultivation with agitation and developing a new reactor such as an airlift reactor, have been attempted [6]. On the other hand, the chemical mutagenesis of *A. xylinum* has also been attempted to select a mutant strain that produces more BC than the wild strain [20].

A. xylinum BPR2001 synthesizes not only water-insoluble BC, but also water-soluble polysaccharide acetan [12]. The chemical structure [9] and biosynthetic pathway [11] of acetan are of interest, however, the role of acetan in BC production has not yet been clarified.

We generated the mutant EP1 from *A. xylinum* BPR2001 by disruption of the *aceA* gene, and speculated that BC production by the mutant EP1 might increase, because this mutant EP1 does not synthesize the byproduct acetan, and the energy produced from the carbon source may be used in the BC biosynthetic pathway. Furthermore, UDP-glucose is a direct precursor in the biosynthetic pathway of both BC and acetan, and the supply of UDP-glucose in the mutant EP1 may increase the BC synthetic pathway. However, the mutant EP1 produced less BC than the wild-strain in the 10-l jar fermentor cultivation (Fig. 1). These results indicate that the metabolic pathway of BC production is not associated with that of acetan synthesis. The total numbers of the wild and mutant cells were almost identical, but the number of the free wild-type cells was more than that of the mutant EP1 cells. Therefore, we speculate that the difference in the free cell number is associated with BC production, because free cells are responsible for active BC production.

Effect of Cell Activity on BC Biosynthesis

ATP concentration and OUR were measured to investigate the cellular activity of the two strains [21]. In a catabolic reaction, *A. xylinum* degrades the carbon source to produce smaller molecules such as pyruvate and acetate as well as energy. On the other hand, the energy generated is used to drive an anabolic reaction, which produces BC and acetan. *A. xylinum* produces chemical energy mainly via

fructose glycolysis in CSL-Fru medium. Fructose is metabolized to fructose-6-phospate (F6P) and glucose-6-phospate (G6P), and G6P is converted to BC and acetan by phosphoglucomutase (PGM), or to carbon dioxide and water, or to gluconate and other organic acids by glucose-6-phosphate dehydrogenase (G6PD). In the present study, there were no significant differences observed in OUR (Fig. 2B) or in ATP concentration (Fig. 2C) between the two strains, and the total cell numbers of the two strains were similar. Since ATP concentration of the mutant EP1 was maintained at the same level as that of the wild-type strain, the energy produced by the mutant EP1 could be utilized for synthesis of substances other than acetan. This speculation suggests that more carbon dioxide or gluconate is produced by the mutant than by the wild-type strain. Ishikawa *et al.* [22] reported that BC production and cell growth of the mutant generated by chemical mutagenesis increased with increasing concentrations of intracellular high-energy compounds such as AMP, ADP, and ATP [22, 23]. However, the disruption of *aceA* did not correlate with the change in the amount of ATP for use in BC synthesis. From these results, we speculate that the increase in BC production by the wild-type strain is not affected by biochemical or genetical factors of acetan production.

Effect of Viscosity on BC Production

Acetan secreted in the medium by BPR2001 made solutions viscous (Fig. 3), thus leading to poor oxygen transport. Consequently, it seems likely that acetan adversely affects cell growth and the rate of BC production. However, BC production by the wild-strain was about 28% greater than that of the acetan-nonproducing mutant EP1. Thus, an increase of viscosity in the culture broth due to acetan production appears to have a positive effect on BC production. Actually, an increase of viscosity in the culture broth accelerated BC production (Fig. 1) and formation of relatively smaller and uniform BC pellets (Fig. 4). Comparison of the BC pellets from the wild-strain and the mutant EP1 shows obvious differences: the sizes of the BC pellets produced by the wild-strain were smaller and more homogeneous than those of the mutant EP1. An increase in the amount of smaller BC pellets increases the number of free cells (Fig. 2A), thus accelerating fructose consumption (Fig. 1) and BC production.

To elucidate the role of acetan in *A. xylinum* BPR2001, agar was added to the EP1, instead of acetan as a water-soluble polysaccharide, and BC production was measured. The relative viscosities of CSL-Fru media containing agar were 1.06, 1.57, and 4.17 in 0, 0.2, and 0.4% (w/v) agar concentrations, respectively. The BC production and fructose consumption by the EP1 in CSL-Fru medium, containing agar in the range of 0 to 0.4% (w/v), are shown in Fig. 5. When agar was added to the culture medium, fructose consumption was enhanced, and the maximum BC production

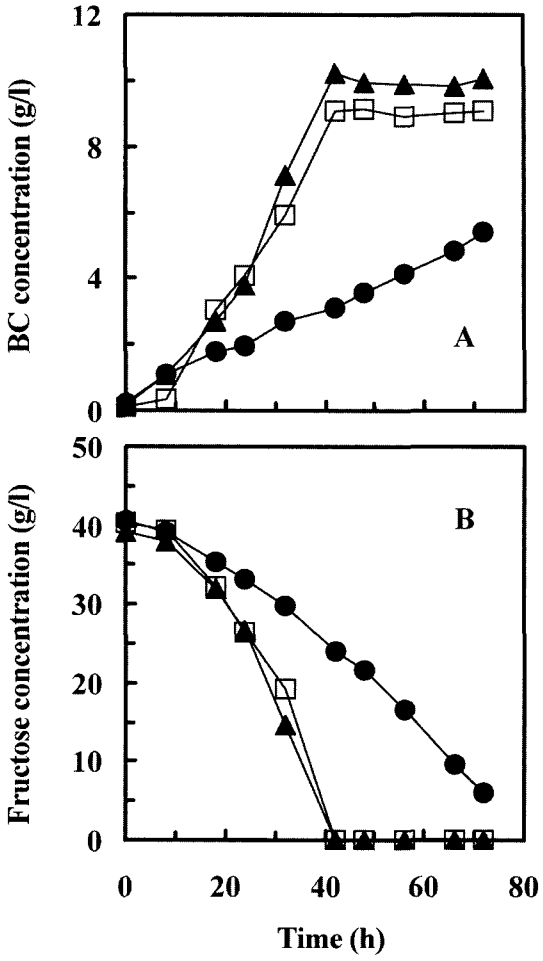

Fig. 5. Bacterial cellulose production and changes in fructose consumption by acetan-nonproducing mutant EP1, when agar was supplementated in CSL-Fru medium.
Agar supplementation concentrations of 0% (closed circles), 0.2% (open squares), and 0.4% (closed triangles) from the start of cultivation.

by the EP1 increased to 10.2 g/l at 0.4% agar concentration, which is 1.9-fold higher than 5.4 g/l in the control medium without agar addition. From the industrial point of view, acetan is not practically applicable, because of difficult purification. However, easily available agar can be replaced with acetan, because a higher viscosity can be maintained from the start of experiment. Furthermore, since BC production was increased and a shorter period of cultivation time was required to reach a maximum BC concentration, we conclude that acetan production by *A. xylinum* BPR2001 is closely associated with BC production, evidenced by the changes in the rheological properties of the medium.

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